Leg lengthening and glycosaminoglycans in the rabbit knee

Eiichi Nakamura, Hiroshi Mizuta, Yutaka Otsuka, Yoshihiko Mizumoto and Katsumasa Takagi

We investigated the effects of tibial lengthening by callotasis on glycosaminoglycan (GAG) metabolism of the knee articular cartilage in 30 rabbits. The distraction rate was 1 mm per day. On the right side, the daily distraction was in 2 steps, while on the left it was in 120 steps. The animals were divided into 3 subgroups based on length gain; 10, 20, and 30 percent, respectively. The knee joint fluid and medial tibial cartilage were examined by quantitative analysis of the GAG content and/or synthesis. The immunoreactivity for chondroitin sulfate in the cartilage was also examined by immunohistochemistry. For all length gains, the GAG concentration in the synovial fluid was higher on both sides than in controls, with no difference between sides. The GAG content and synthesis in the cartilage on the 2-step side decreased gradually with increasing length. On the 120-step side, the content did not differ from control values in any length gain, and the level of synthesis at 20 and 30 percent lengthening was higher than the control level. Our findings indicate that the alterations in GAG metabolism are attributable to increased mechanical stress on the articular cartilage, suggesting a moderate increase on the 120-step side compared to an excessive one on the 2-step side.

Department of Orthopedics, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860, Japan
Tel +81 96-344 2111. Fax -362 0532
Submitted 94-04-09. Accepted 94-10-14

Gradual distraction techniques for limb lengthening carry a risk of exerting increased mechanical stress on the articular surfaces of adjacent joints, leading to cartilage damage (de Pablos and Cañadell 1989, Paley 1990). However, the nature of this damage is not well known. We have previously shown that increasing the frequency of distraction can help to prevent degenerative morphological changes in articular cartilage in rabbit knee joints during tibial lengthening by callotasis (Nakamura et al. 1993).

In this study, we investigated the effect of 2 different frequencies of distraction on the articular cartilage glycosaminoglycan (GAG) metabolism in rabbit knee joints during tibial lengthening.

Animals and methods

30 skeletally mature New Zealand white rabbits were used. Under Nembutal® anesthesia, an incision was made on the medial aspect of the tibia, and 2.4-mm fixation pins were inserted at right angles to the diaphysis. A unilateral external fixator was applied after transverse osteotomy just below the tibiofibular junction. Both tibiae were operated on in the same manner. Distraction at a rate of 1 mm per day was started on the day after operation. On the right side, the frequency of distraction was 2 steps per day (0.5 mm every 12 h) by hand, while on the left it was 120 steps per day (0.0083 mm every 12 min) by autodistractor, as described in a previous study (Nakamura et al. 1993).

The 30 animals were divided into 3 equal subgroups subjected to 10, 20, and 30 percent increases in length. At the end of the experiment, the animals were killed with Nembutal®.

The control values were obtained from 15 age-matched rabbits not subjected to surgery. From each of their knees, a total of 30 normal joint fluid samples were aspirated as described below. From 5 of those knees, selected at random, 5 medial tibial cartilage slices were used for the determination of GAG content and for immunohistochemical examination, as described below. For the analysis of ^{35}S-GAG synthesis, 5 medial tibial cartilage slices from another 5 knees, also selected at random, were obtained under sterile conditions for use as control samples.

Tissue preparation

In 15 of the rabbits (5 from each subgroup), the knee joint fluid and articular cartilage were obtained from both knees for GAG analysis. Immediately after death, 1 mL of sterile sodium chloride solution was injected into both knee joints and the knees were taken 10 times through a full range of motion. The joint fluid was then aspirated and centrifuged at 10,000g for 10 min. The supernates were divided equally into 2 plastic tubes, lyophilized, and stored at –80 °C until analysis. Both tibiae were then removed. For biochemical and immunohistochemical examinations, 2 full-depth slices of articular cartilage were obtained from the area not covered by the meniscus on the medial tibial condyle.

In the remaining 15 rabbits (5 from each subgroup), both tibiae were removed under sterile conditions immediately after death. For analysis of ^{35}S-GAG synthesis, 2 full-depth slices of cartilage were shaved from the same region, as described above.

GAG in joint fluid

The concentrations of chondroitin 4-sulfate (C4S), chondroitin 6-sulfate(C6S), and keratan sulfate (KS) were measured as the major GAGs. The concentration of GAG was determined from the sum of the concentrations of C4S, C6S, and KS.

After all lyophilized samples were dissolved in 0.8 mL distilled water, analysis of chondroitin sulfate isomers was performed according to the method of Shinmei et al. (1992). One sample of a duplicate was used for digestion with chondroitinase ABC (Seikagakukogyo, Tokyo, Japan) and ultrafiltered as follows. 50 µL of chondroitinase ABC solution (5 units in 1 mL distilled water), 80 µL of 10 mM sodium acetate buffer (pH 8.0), and 70 µL of distilled water were mixed with 200 µL of dissolved sample. After incubation at 37 °C for 2 h, the mixture was ultrafiltered (molecular weight cutoff 10000). The unsaturated disaccharides in the filtrate were applied to a High Performance Liquid Chromatography system. After the eluant from the column reacted with 2-cyanoacetamide (Aldrich, Milwaukee, WI) through a reaction coil, the effluents were monitored by spectrofluorometry (331 nm excitation and 383 nm emission). The peak areas corresponding to the 4-sulfated and 6-sulfated unsaturated disaccharides were calculated using the integrator.

From the other solution, the concentration of KS was determined by enzyme-linked immunosorbent assay (ELISA), as described by Kongtawelert and Ghosh (1990). The concentration of GAG, determined above, is considered to reflect the total amount of GAG in the knee joint fluid.

GAG in articular cartilage

Cartilage samples were maintained in phosphate buffer saline (PBS), and GAG analysis was performed using dimethyl methylene blue (Farndale et al. 1982). GAG content was adjusted for differences in the wet weight of the cartilage.

Immunohistochemistry

Cartilage slices were fixed with periodate-lysine-paraformaldehyde and sectioned at 6 µm with a cryostat. The sections were digested with chondroitinase ABC (0.5 units per mL) in acetate buffer (pH 8.0) at 37 °C for 1 h. After blocking the endogenous peroxidase activity and the non-specific reaction, all sections were then incubated at 37 °C for 1 h with the 2-B-6 (1:100) or 3-B-3 (1:100) monoclonal antibodies (Seikagakukogyo, Tokyo, Japan) which recognized the 4-sulfated and 6-sulfated unsaturated disaccharides, respectively (Caterson et al. 1985). Then the sections were washed in PBS and treated with a horseradish peroxidase-conjugated secondary antibody. Staining for peroxidase was performed with 0.5 mg/mL 3,3'-diaminobenzidine and 0.03% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6, for 10 min.

GAG metabolism in articular cartilage

One slice of the cartilage was cultured in a tissue culture plate containing Ham's F-12 nutrient mixture as described by Palmoski and Brandt (1979). After preincubation for 24 h, this cartilage was labeled with $^{35}SO_4$ (20 µCi/mL, carrier-free, NEN) for 4 h. Then the medium was removed and the cartilage slice rinsed. The medium and washes were combined and dialyzed against distilled water for 72 h at 4 °C in Spectrapor 3 dialysis tubing (Spectrum). The rinsed cartilage was digested overnight at 56 °C in 1 mL of 0.067 M phosphate buffer solution (pH 7.4) containing pronase (1 mg/20–30 mg tissue), then the digests were dialyzed as above. After dialysis of the medium and washes, 1 mL of the retentates was added to 4 mL of AQUASOL-2 (NEN) and counted in an LS-3050 liquid scintillation spectrometer (Aroka). The same was done to the digests. ^{35}S-GAG synthesis was determined from the sum of the nondialyzable ^{35}S in the medium and pronase digest, and was expressed as cpm per mg wet tissue.

The DNA content (µg per wet mg weight) of another slice was determined using fluorometry, as described by Oegema et al. (1984). Net ^{35}S-GAG synthesis was analyzed as cpm per DNA µg.

Statistics

ANOVA was used to evaluate the influence of length gain and the daily frequency of distraction. The non-

Table 1. Results of knee joint GAG-analysis (Mean *SD*)

Percent increase in length	GAG content in tibial cartilage (μg/mg wet weight)		^{35}S-sulfate incorporation of cartilage explants (thousand cpm / μg DNA)		GAG concentrations in synovial fluid (μg/mL)	
	2 steps	120 steps	2 steps	120 steps	2 steps	120 steps
Control	17.3 *3.6*		8.1 *0.8*		3.5 *1.7*	
10, n 5	11.5 [a] *4.6*	13.1 *4.3*	9.1 *2.0*	8.8 *3.2*	10.7 [a,c] *6.6*	6.5 *4.8*
20, n 5	7.1 [b,c] *1.0*	12.6 *3.3*	7.2 [a,c] *2.3*	13.9 [b] *1.3*	11.7 [b] *8.1*	7.9 [b] *3.7*
30, n 5	5.1 [b,c] *2.7*	12.8 *3.6*	4.5 [b,c] *2.0*	12.8 [b] *2.4*	13.7 [b] *4.1*	13.6 [b] *6.8*

Difference [a]($P < 0.05$), [b]($P < 0.01$) from control value.
Difference [c]($P < 0.05$), [d]($P < 0.01$) from value for 120-step side.

parametric Kruskall-Wallis test was used to compare the 4 groups comprising 3 subgroups in the same step and the control group, as also was the ANOVA. When significant differences were found, the Mann-Whitney U-test was used. To evaluate differences between sides at the same length gain, the Pratt test was used. $P < 0.05$ was considered significant.

Results

Contents of GAG in the cartilage

For all length gains, the mean GAG contents of the cartilage on the 2-step side were lower than those in the control cartilage. On the 120-step side, however, no values for any length gains differed from the controls. At 20 and 30 percent length gains, the mean GAG contents on the 2-step side were lower than those on the 120-step side (Table 1).

Quantification of ^{35}S-GAG synthesis

Net ^{35}S-GAG synthesis at 10 percent length increase on both sides was not significantly different from the control value. At 20 and 30 percent increases, net ^{35}S-GAG synthesis on the 2-step side showed 0.78- and 0.56-fold decreases, while those on the 120-step side showed 1.7- and 1.5-fold increases, respectively, compared with the control value. At 20 and 30 percent length gains, net ^{35}S-GAG synthesis on the 2-step side was lower than those on the 120-step side (Table 1).

Immunoreactivities for chondroitin sulfate

Compared to control sections, the immunoreactivity for C4S and C6S in the sections from the 2-step side became weaker in the extracellular matrix of all zones as the length increased. On the 120-step side, this immunoreactivity in the interterritorial matrix remained similar to that in the control cartilage and stronger in pericellular matrix of the middle zones (Figure 1).

Concentrations of GAG in joint fluid

All values for both sides were higher than in the control knee (3.5 μg/mL), except the concentration at 10 percent increase on the 120-step side. No significant differences in these values were found between the sides (Table 1).

Discussion

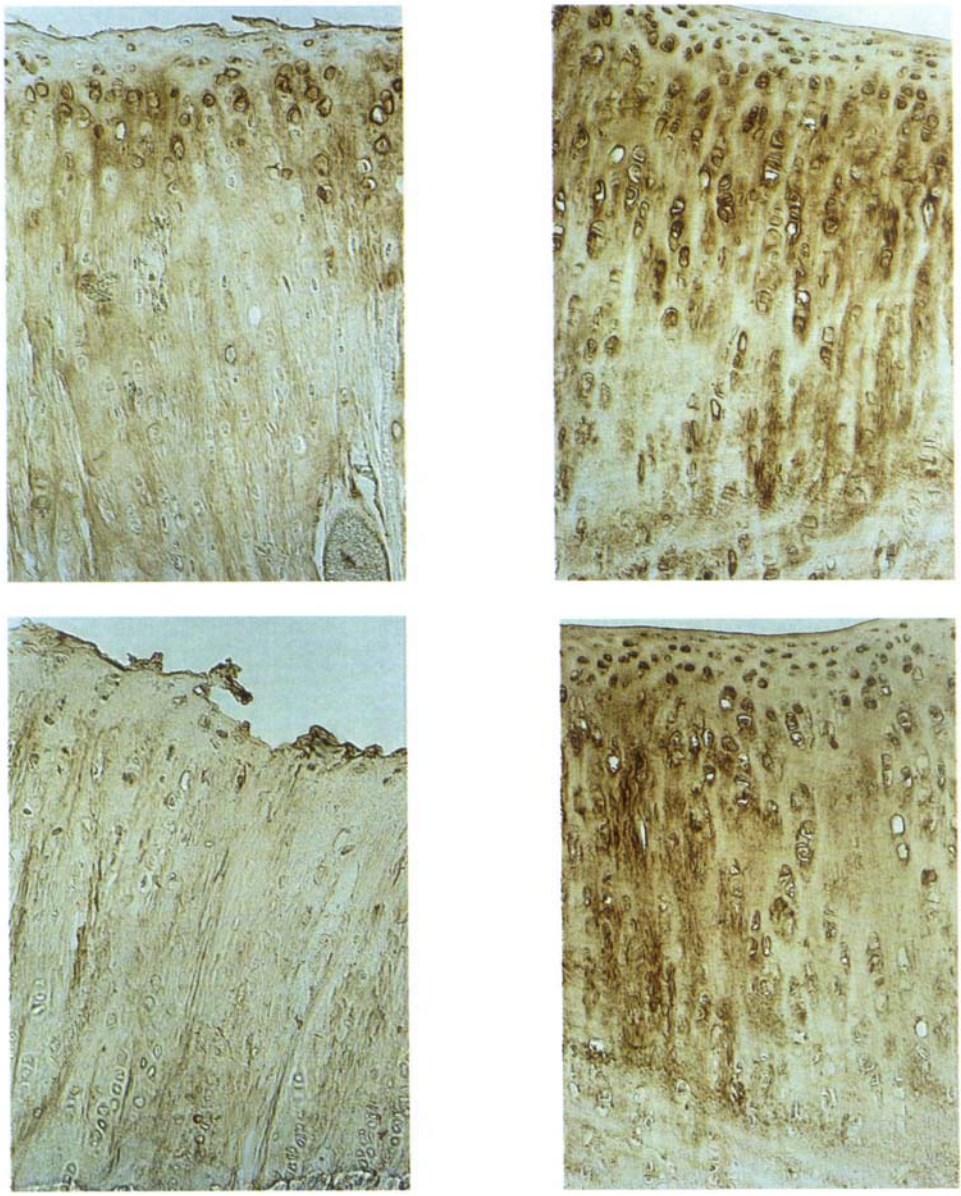
Mechanical stress is important in modulating the metabolic activity of the chondrocyte and in maintaining the structure of the matrix. Stress below the normal range leads to deterioration of articular cartilage. In contrast, increased mechanical stress does not evoke a uniform pattern of response. Excessive stress causes a loss of GAG content and a decrease in synthesis (Hall et al. 1991, Sah et al. 1991, Kiviranta et al. 1992), while a moderate stress sometimes leads to an increase in GAG content and to an increase in synthesis (Palmoski and Brandt 1984, Säämänen et al. 1989).

On the 2-step side, the GAG contents and synthesis decreased with increasing length. On the 120-step side, the GAG content remained normal, while synthesis tended to increase. Chondroitin sulfate staining produced similar data. The difference in the GAG content and synthesis on the 2 sides in this study may be considered to reflect the difference in the mechanical stress on the articular cartilage: the stress exerted by the 2-step distraction is excessive, while that on the 120-step side is moderate.

Sah et al. (1991) reported that the amount of loss in existing GAG from the matrix due to mechanical stress was directly proportional to the magnitude of

Figure 1. Immunohistochemical demonstration of chondroitin sulfate in sections from the medial tibial condyle on both sides at 30 percent length gain (×560).

2-step-side **120 step-side**



Immunoreactivity for C4S (above) and C6S (below) in the 2-step-side sections was relatively weak in the extracellular matrix of all zones, but slightly reactive for C4S in the transitional zone.

Immunoreactivity for C4S (above) and C6S (below) in the 120-step-side sections. Both reactivities were detected in all interterritorial regions and more intensely in the pericellular matrix of the middle zones.

the stress to which it is subjected. In our study, however, the GAG concentration in the knee joint fluids showed no difference between sides. The newly synthesized proteoglycans produced as a consequence of increased mechanical stress have a lower affinity for hyaluronic acid (Säämänen et al. 1989). Therefore, some of them are prone to release into the synovial fluid (Caroll et al. 1992). Thus, the discrepancy between our results and those of Sah et al. (1991) may be that on the 120-step side some of the newly

synthesized GAG adds to the amount of loss in the existing GAG, while on the 2-step side the GAG concentration represents the amount of the existing GAG loss.

In addition to causing direct damage to the structure of the matrix, the increased mechanical stress can affect the chondrocyte metabolic activity, thus altering the mechanical properties of the cartilage due to a change in matrix constituents. In our study, the loss of GAG content in the cartilage on the 2-step side is considered to lead to a decreased compressive stiffness (Kempson et al. 1970), which reduces the ability to resist further mechanical stress. The degenerative change in the cartilage on the 2-step side, shown in our earlier histological study (Nakamura et al. 1993), may be partly attributable to the loss of GAG content, as well as to direct damage to the structure of the matrix due to mechanical stress.

Acknowledgements

We would like to thank Drs. Kazuki Takashima and Shinji Andou, in the Department of Material Development and Resource Engineering, Kumamoto University, and Hiroshi Maeda, Kazuo Okamura and Kyosuke Miyazaki, Carbohydrate Analysis Center, Seikagaku Corporation, for their skillful technical assistance.

References

Caroll G J, Bell M C, Laing B A, McCappin S, Blumer C, Leslie A. Reduction of the concentration and total amount of keratan sulphate in synovial fluid from patients with osteoarthritis during treatment with piroxicam. Ann Rheum Dis 1992; 51 (7): 850–4.

Caterson B, Christner J E, Baker J R, Couchman J R. Production and characterisation of monoclonal antibodies directed against connective tissue proteoglycans. Fed Proc 1985; 44 (2): 386–93.

de Pablos J, Cañadell J. Methods of bone lengthening and their applications. Servicio de Publicaciones de la Universidad de Navara, Pamplona 1989.

Farndale R W, Sayers C A, Barrett A J. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 1982; 9 (4): 247–8.

Hall A C, Urban J P G, Gehl K A. The effects of hydrostatic pressure on matrix synthesis in articular cartilage. J Orthop Res 1991; 9 (1): 1–10.

Kempson G E, Muir H, Swanson S A V, Freeman M A R. Correlations between stiffness and the chemical constituents of cartilage on the human femoral head. Biochim Biophys Acta 1970; 215: 70–7.

Kiviranta I, Tammi M, Jurvelin J, Arokoski J, Säämänen A-M, Helminen H J. Articular cartilage thickness and glycosaminoglycan distribution in the canine knee joint after strenuous running exercise. Clin Orthop 1992; 283: 302–8.

Kongtawelert P, Ghosh P. A new sandwich-ELISA method for the determination of keratan sulphate peptides in biological fluids employing a monoclonal antibody and labelled avidin biotin technique. Clin Chim Acta 1990; 195: 17–26.

Nakamura E, Mizuta H, Sei A, Takagi K. Knee articular cartilage injury in leg lengthening. Histological studies in rabbits. Acta Orthop Scand 1993; 64 (4): 437–40.

Oegema T R, Carpenter B J, Thompson R C. Fluorometric determination of DNA in cartilage of various species. J Orthop Res 1984; 1 (4): 345–51.

Paley D. Problems, obstacles, and complications of limb lengthening by Ilizarov technique. Clin Orthop 1990; 250: 81–104.

Palmoski M J, Brandt K D. Effect of salicylate on proteoglycan metabolism in normal canine articular cartilage in vitro. Arthritis Rheum 1979; 22 (6): 746–54.

Palmoski M J, Brandt K D. Effects of static and cyclic compressive loading on articular cartilage plugs in vitro. Arthritis Rheum 1984; 27 (6): 675–81.

Sah R L-Y, Doong J-Y H, Grodzinsky A J, Plaas A H K, Sandy J D. Effects of compression on the loss of newly synthesized proteoglycan and proteins from cartilage explants. Arch Biochem Biophys 1991; 286 (1): 20–9.

Shinmei M, Miyauchi S, Machida A, Miyazaki K. Quantitation of chondroitin 4-sulfate and chondroitin 6-sulfate in pathologic joint fluid. Arthritis Rheum 1992; 35 (11): 1304–8.

Säämänen A-M, Tammi M, Kiviranta I, Jurvelin J, Helminen H J. Levels of chondroitin-6-sulfate and non-aggregating proteoglycans at articular cartilage contact sites in the knees of young dogs subjected to moderate running exercise. Arthritis Rheum 1989; 32 (10): 1282–92.